Interleukin-4 Causes Susceptibility to Invasive Pulmonary Aspergillosis through Suppression of Protective Type I Responses

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Aspergillus fumigatus, **an opportunistic fungal pathogen, causes multiple allergic and nonallergic airway diseases. Invasive pulmonary aspergillosis (IPA) is a nonallergic, life-threatening disease of immunocompromised patients. In a murine model of IPA, interleukin (IL)–4–deficient (IL-4$^{-/-}$) BALB/c mice were used to examine the role of IL-4 in lung pathology and immune responses. IL-4$^{-/-}$ mice were more resistant than wild-type mice to infection caused by multiple intranasal injections of viable *A. fumigatus* conidia. Resistance was associated with decreased lung inflammatory pathology, impaired T helper (Th)–2 responses (including lung eosinophilia), and an IL-12–dependent Th1 response. In contrast, development of host-detrimental antifungal Th2 cells occurred in IL-12$^{-/-}$ and interferon-$\gamma^{-/-}$ mice and in IL-4$^{-/-}$ mice when subjected to IL-12 neutralization. These results demonstrate that IL-4 renders mice susceptible to infection with *A. fumigatus* by inhibition of protective Th1 responses. IL-4 appears to have a distinct role in the pathogenesis of allergic and nonallergic lung diseases caused by the fungus.**

Aspergillus fumigatus is a ubiquitous and opportunistic fungal pathogen of humans and animals. It is associated with a wide spectrum of diseases, ranging from benign colonization of the lung and allergies to life-threatening diseases such as invasive pulmonary aspergillosis (IPA) or allergic bronchopulmonary aspergillosis (ABPA) [1]. A variety of underlying conditions, including impaired immune status, contribute to development of aspergillosis [2]. In moderately immunocompromised persons, a chronic necrotizing aspergillosis may be a discrete clinical entity [3]. However, among immunocompromised persons, IPA, characterized by hyphal invasion and destruction of pulmonary tissue, is the most common manifestation of *Aspergillus* infection [1, 4]. It has been suggested that cells of the innate immune system may play an important role in the defense against IPA [5]. Resident alveolar macrophages ingest and kill resting conidia, largely through nonoxidative mechanisms, while neutrophils use oxygen-dependent mechanisms to attack hyphae germinating from conidia that escape macrophage surveillance [6]. Major recognized risk factors for disease are defects in phagocyte respiratory burst, such as those occurring in chronic granulomatous disease [7, 8]; cortisone-induced suppression of macrophage conidiocidal activity [9, 10], and neutropenia [11]. Increased risk for a chronic form of IPA, which is independent of neutropenia and corticosteroid therapy, has been observed in persons infected with human immunodeficiency virus [12], who also showed a defective effector activity of neutrophils against *A. fumigatus* [13].

More recent evidence in humans indicates that a dysregulated production of T helper (Th) cell cytokines may contribute to the pathogenesis of IPA [14–16]. In a murine model of IPA, we showed that production of Th1 and Th2 cytokines differed in mice resistant or susceptible to the infection [17]. Resistance to infection and development of protective immunity were associated with high levels of tumor necrosis factor (TNF)–α and interleukin (IL)–12, an efficient innate antifungal effector function, and with detection of interstitial lung lymphocytes producing interferon (IFN)–γ. In contrast, production of IL-4 and IL-10 by interstitial CD4 Th2 lymphocytes was associated with disease progression, and local IL-4 or IL-10 neutralization improved the infection [17].

IL-4 is the primary determinant of differentiation of CD4 T cells into Th2 cytokine-producing cells in fungal infections [18]. In addition to its pivotal role in CD4 T cell differentiation, IL-4 mediates inflammatory pathology in allergic lung diseases [19]. Despite substantial evidence for the importance of IL-4 in these diseases, much controversy remains on its specific

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Animal care and procedures conformed with national and international laws and policies.

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pathogenetic role [20–22]. In the case of exposure to *A. fumigatus* antigens, a further complication arises from clinical and experimental findings indicating that both Th2 [23, 24] and Th1 [25, 26] cells contribute to the pathogenesis of the ensuing lung diseases. To definitively describe the role of IL-4 in the infection and lung pathogenesis of airway disease caused by the fungus, we evaluated the course and outcome of IPA, lung pathology, and parameters of local innate and adaptive Th immunity in IL-4–deficient (IL-4$^{-/-}$) mice on exposure to *A. fumigatus* conidia.

Materials and Methods

Mice. We used female 8–10-week-old inbred DBA/2 (*H-2*^{d}) mice (Charles River, Calco, Italy). Breeding pairs of homozygous IL-4$^{-/-}$, IL-5–deficient (IL-5$^{-/-}$), and IFN-γ–deficient (IFN-γ$^{-/-}$), generated as described elsewhere [27–29], were backcrossed for several generations (6 or 7) to BALB/c at the Basel Institute for Immunology. Breeding pairs of homozygous IL-12p40–deficient (IL-12p40$^{-/-}$) and BALB/c wild-type mice were provided by L. Adorini (Hoffmann-LaRoche, Milan, Italy). Knockout and wild-type control mice were maintained under specific pathogen-free conditions in the Medical School of Perugia animal facility. Mice 8–10 weeks old of both sexes were used.

Microorganism, culture conditions, and infection. The *A. fumigatus* strain, derived from a person who died of pulmonary aspergillosis, was obtained at the Infectious Diseases Institute of the University of Perugia. The microorganism was grown for 4 days at room temperature on Sabouraud dextrose agar (Difco Laboratories, Detroit) supplemented with chloramphenicol. Abundant conidia were elaborated under these conditions. Conidia were removed by washing the slant culture with 5 mL of 0.025% Tween 20 in saline and gently scraping the conidia from the mycelium with a plastic pipette (or by vigorous shaking). Cell debris was allowed to settle by gravity, and the suspension was decanted into 50-mL plastic conical tubes. After extensive washing of the conidia with saline, conidia were counted and diluted to the desired concentrations. The viability of the conidia was >95%, as determined by serial dilution and plating of the inoculum on Sabouraud dextrose agar. For intranasal (inl) infection, mice were lightly anesthetized by inhaled diethyl ether before instillation for 3 consecutive days with a suspension of 2×10^7 conidia/20 μL of saline slowly applied to the nostrils with a micropipette with a sterile disposable tip. The animals were held upright until the suspension was completely inhaled and normal breathing resumed [17]. Mice dying of fungal infection were routinely necropsied for histopathologic confirmation of invasive aspergillosis. For histology, tissues were excised and immediately fixed in formalin. Sections (3–4 μm) of paraffin-embedded tissues were stained by periodic acid–Schiff or Giemsa.

Chitin assay. For the quantification of fungal growth in the organs, we used the chitin assay as described elsewhere [17, 30]. Results are expressed as micrograms of glucosamine/organ. Positive control included serially diluted D(+)-glucosamine (Sigma, St. Louis). The glucosamine content of lungs from uninfected mice was used as negative control; this ranged from 0.80 to 2.25 μg of glucosamine/organ.

Collection of bronchoalveolar lavage (BAL) fluid. Lungs were lavaged thoroughly with 0.5 mL of PBS injected via the trachea, as described elsewhere [17]. The lavage fluid was centrifuged, and the supernatant was removed and immediately stored at −80°C until analyzed for cytokine and IgE contents.

In vivo treatments. For immunosuppression, mice were injected intraperitoneally (ip) with 150 mg/kg of cyclophosphamide (Sigma) 3 days before challenge. For B7-1 and B7-2 blockade in vivo, mice were given inl 100 μg of affinity-purified hamster anti-mouse B7-1 (clone 16-10A1) [31] and/or rat anti-mouse B7-2 (clone GL1) [32] in 50 μL of saline 4 h before fungal challenge. Control animals were administered 100 μg of hamster IgG (Zymed Laboratories, South San Francisco) or the isotype-matched rat IgG2a (GL117). Polyclonal sheep anti–IL-12 antibodies (Genetics Institute, Cambridge, MA) or control antibodies (Zymed) were given ip (500 μg) 4 h before the fungal inoculation. Endotoxin was removed from all solutions with Detoxi-gel (Pierce Chemical, Rockford, IL).

Isolation, phenotypic analysis, and cell culture. Interstitial lung lymphocytes were isolated as described elsewhere [17]. The pulmonary vasculature was perfused with 5 mL of PBS containing 100 U/mL heparin to eliminate peripheral blood cells, and the lungs were removed, minced, and incubated for 90 min at 37°C in digestion buffer containing 0.7 μg/mL collagenase (Sigma) and 30 μg/mL type IV bovine pancreatic DNase I (Sigma). Large particulate matter was removed by passing the cell suspension through a small, loose, nylon wool plug, and lymphocytes were enriched over a single-step ficoll gradient. Flow cytometric analysis of lung T cells revealed that interstitial lymphocytes consisted of αβTCR-bearing CD3 cells (62% and 55% in mutant and wild-type mice, respectively); γδTCR-bearing CD3 cells (11% and 9%); B220^{+} cells (27% and 25%); CD3^{+}CD4^{high} (8% and 8%), CD3^{+}CD45RB^{low} (23% and 33%), and CD3^{+}CD28^{+} cells (21% and 17%). Cells were resuspended in RPMI containing 10% fetal calf serum, 2-mercaptoethanol (50 m*M*), sodium pyruvate (1 m*M*), HEPES (10 m*M*), and gentamycin (50 μg/mL) and were plated at a concentration of 2×10^5 in 96-well microtiter plates coated with 12 μg/mL hamster anti-mouse CD3 monoclonal antibody (MAb; clone 145-2C11; PharMingen, San Diego). After 48 h of culture, supernatants were removed, and cytokine production was determined by specific ELISAs. Purified lung macrophages and neutrophils were obtained by 2-h plastic adherence or by positive selection with the anti-granulocyte MAb RB6-8C5, respectively, as described elsewhere [17, 33].

Cytokine and IgE assays. The levels of IFN-γ, IL-4, IL-5, and IL-10 in culture supernatants of activated cells were determined by cytokine-specific ELISAs using pairs of anti-cytokine MAbs, as described elsewhere [17, 30]. The antibody pairs used were as follows, listed by capture/biotinylated detection: IFN-γ, R4-6A2/XMG1.2; IL-4, BVD4-1D11/BVD6-24G2; IL-5, TRFK-5/TRFK-4; IL-10, JES5-2A5/SXC-1 (PharMingen). For IL-12p70 measurement, a modified antibody capture bioassay was used [17, 30]. Cytokine titers were calculated by reference to standard curves constructed with known amounts of recombinant cytokines from PharMingen, except IL-12, which was from Genetics Institute. A micro-ELISA procedure was used to quantitate total IgE in the BAL fluid [17]. Each sample was assayed in triplicate ⩾3 times.

Hyphal damage and conidiocidal assays. A colorimetric MTT assay [17] was used to study *Aspergillus* hyphal damage by lung

cells. Purified neutrophils (10^6) were added to 10^5 conidia that had been cultured at 37°C in 5% CO_2 with fetal calf serum for 16–18 h in 96-well flat-bottomed microtiter plates (Costar, Cambridge, MA), by which time >95% of the conidia had germinated to hyphae (~150–200 μm long). After 2 h at 37°C with occasional shaking, the supernatants were aspirated, effector cells were lysed by adding 0.5% sodium deoxycholate, and hyphae viability was determined by MTT staining. For the conidiocidal activity, 10^5 conidia were mixed with 10^6 purified alveolar macrophages for 4 h in 96-well flat-bottomed microtiter plates (Costar), and colony-forming units (cfu) were counted as described elsewhere [17]. The percentage of cfu inhibition (mean ± SE) was determined as follows: % colony-formation inhibition = [100 − (cfu experimental group/cfu control cultures)] × 100.

Flow cytometry. Total lung cells were sequentially reacted with saturating amounts of phycoerythrin-conjugated anti-CD3 (clone 500A2) and fluorescein isothiocyanate (FITC)–conjugated anti-CD4 (clone RM4-5), anti-CD8 (clone 53-6.7), anti-$\alpha\beta$TCR (clone H57-597), anti-$\gamma\delta$TCR (clone GL3), anti-CD44 (clone IM7), anti-CD45RB (clone 23G2), and anti-CD28 (clone 37.51) MAbs. Total lung cells or B cells, purified by panning on antiimmunoglobulin-coated plates, were reacted with saturating amounts of FITC-conjugated anti–B7-1 (clone 16-10A1) and anti–B7-2 (clone GL1) MAbs from PharMingen. Cells were analyzed with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) by use of Cell Quest software (Becton Dickinson) for Macintosh. Nonviable cells were excluded from analysis by accepted procedures involving propidium iodide and narrow-forward-angle light-scatter gating. The percentage of positive phagocytes (mainly neutrophils and macrophages) was obtained on cells electronically gated by forward light and scatter analysis, as described elsewhere [34]. The percentage of positive cells obtained with an irrelevant antibody was used as the background value and was always subtracted.

RNA preparation and reverse-transcriptase (RT) polymerase chain reaction (PCR). Total lung cells were subjected to RNA extraction by the guanidium thiocyanate–phenol-chloroform procedure, as described elsewhere [34, 35]. In brief, 5 μg of total RNA was reverse transcribed into cDNA by use of M-MLV RT (GIBCO BRL Life Technologies, Gaithersburg, MD). The cDNA was then amplified with specific primers for murine IL-12 receptor β1 and β2 (IL-12Rβ1 and IL-12Rβ2) and hypoxanthine-guanine phosphoribosyl transferase (HPRT), the latter as an internal control. The IL-12Rβ1–, IL-12Rβ2–, and HPRT–specific primers were the same as described elsewhere [34].

Amplifications were performed in 2 m*M* $MgCl_2$, 50 m*M* KCl, 10 m*M* Tris-HCl (at pH 8.3), 0.2 m*M* of each deoxynucleotide triphosphate, 1 μM of each primer, and 2.5 U of AmpliTaq polymerase (Perkin-Elmer Cetus, Norwalk, CT). The cDNA was amplified in an automated thermal cycler (Perkin-Elmer Cetus) at 94°C for 60 s, 60°C for 60 s, and 72°C for 60 s. Amplification was stopped at 35 cycles. The HPRT primers were used as a control for both reverse transcription and the PCR reaction and for comparing the amount of products from samples obtained with the same primers. The PCR fragments were analyzed by 1.5% agarose gel electrophoresis, stained with 0.5 mg/mL ethidium bromide, and visualized under UV transillumination.

Statistical analysis. Survival data were analyzed by using the Mann-Whitney *U* test; significance was defined as $P < .01$. Student's *t* test was used to determine statistical significance between cytokine production or chitin content; significance was defined as $P < .05$. In vivo groups consisted of 6–10 animals. Unless otherwise specified, the data reported were pooled from 3–5 experiments with similar results. Linear regression analysis was performed using the DeltaGraph program (SPSS, Chicago).

Results

Course of IPA in IL-4$^{-/-}$ and IL-4$^{+/+}$ mice. To assess the susceptibility of IL-4$^{-/-}$ and IL-4$^{+/+}$ mice to IPA, mice either were treated with cyclophosphamide for immunosuppression or were left untreated before multiple inl administrations of 2×10^7 viable *A. fumigatus* conidia and were monitored for mortality, fungal growth in lungs, and lung pathology. The results (table 1) show that the fungal growth in the lungs was significantly reduced in IL-4$^{-/-}$ compared with wild-type mice, although both types of mice survived the infection untreated. When treated with cyclophosphamide, IL-4$^{+/+}$ mice died of the infection, similar to DBA/2 mice, which are highly susceptible to IPA [17]. Lung sections revealed the presence of numerous septate branching hyphae penetrating tissue, with signs of bronchial wall damage, peribronchial necrosis, and numerous infiltrating (predominantly polymorphonuclear) cells (figure 1*A*), including eosinophils, which were scattered throughout the lung parenchyma (E.C., unpublished data). In contrast, cyclophosphamide-treated IL-4$^{-/-}$ mice were resistant to infection and survived. In their lung sections there were few infiltrates (rare small hyphae with a moderate infiltration of inflammatory mononuclear and polymorphonuclear cells in the peribronchial region; figure 1*B*). No eosinophils were found.

To analyze whether eosinophils were responsible for the lung

Table 1. Course and outcome of invasive pulmonary aspergillosis in interleukin (IL)–4$^{-/-}$ and IL-5$^{-/-}$ mice.

			Infection[b]		Chitin content[c]	
Group	Cyclophosphamide[a]	Mice	MST	D/T	1 day	7 days
1	−	IL-4$^{-/-}$	>60	0/8	9.24[d]	0.80[d]
2	−	IL-4$^{+/+}$	>60	0/10	16.72	3.31
3	+	IL-4$^{-/-}$	>60[e]	2/8	12.40[d]	7.45
4	+	IL-4$^{+/+}$	8	10/10	28.44	…
5	+	IL-5$^{-/-}$	9	8/8	32.42	…
6	+	IL-5$^{+/+}$	8	10/10	31.76	…
7	−	DBA/2	>60	1/8	18.65	4.21
8	+	DBA/2	5	8/8	34.51	…

NOTE. IL-4$^{-/-}$, IL-4 deficient; IL-4$^{+/+}$, IL-4 nondeficient; IL-5$^{-/-}$, IL-5 deficient; IL-5$^{+/+}$, IL-5 nondeficient; MST, median survival time (days); D/T, no. of dead mice over total no. of mice infected.

[a] Cyclophosphamide (150 mg/kg) was given intraperitoneally the day before first intranasal (inl) challenge. Minus sign (−), not treated; plus sign (+), treated.

[b] Mice were injected inl with 2×10^7 conidia of *Aspergillus fumigatus* on days 0 and 1 and 2 days later.

[c] Chitin content in lungs, expressed as μg of glucosamine/organ, was determined 1 and 7 days after last fungal inl inoculation (values are mean of 6–8 animals/group from 1 representative experiment). Ellipses (…) indicate data not available.

[d] $P < .05$ (group 1 vs. 2 and group 3 vs. 4 and 5, Student's *t* test).

[e] $P < .01$ (group 3 vs. 4, Mann-Whitney *U* test).

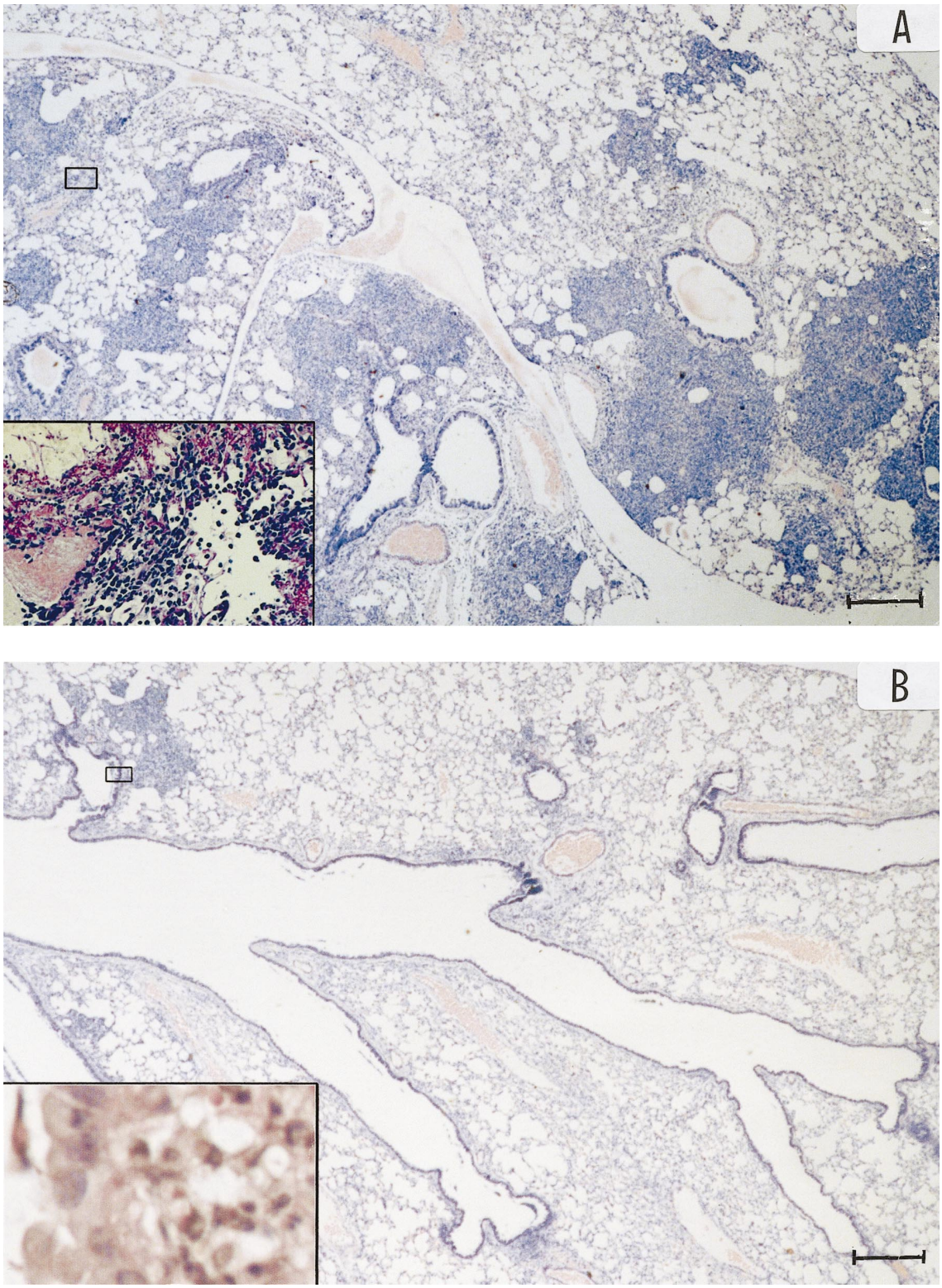

Figure 1. Lung histology of *(A)* immunosuppressed interleukin-4–nondeficient (IL-$4^{+/+}$) and *(B)* –deficient (IL-$4^{-/-}$) mice with invasive pulmonary aspergillosis. Giemsa- and periodic acid–Schiff–stained sections were prepared 1 day after last intranasal inoculation of *Aspergillus fumigatus* conidia. *A,* numerous septate branching hyphae penetrate through tissue with signs of bronchial wall damage, peribronchial necrosis, and numerous infiltrating cells, predominantly polymorphonuclear cells (see inset). Bar = 400 μm; 50 μm in inset. *B,* a few infiltrates and rare small hyphae with moderate infiltration of inflammatory mononuclear cells in peribronchial region. Bar = 400 μm; 100 μm in inset.

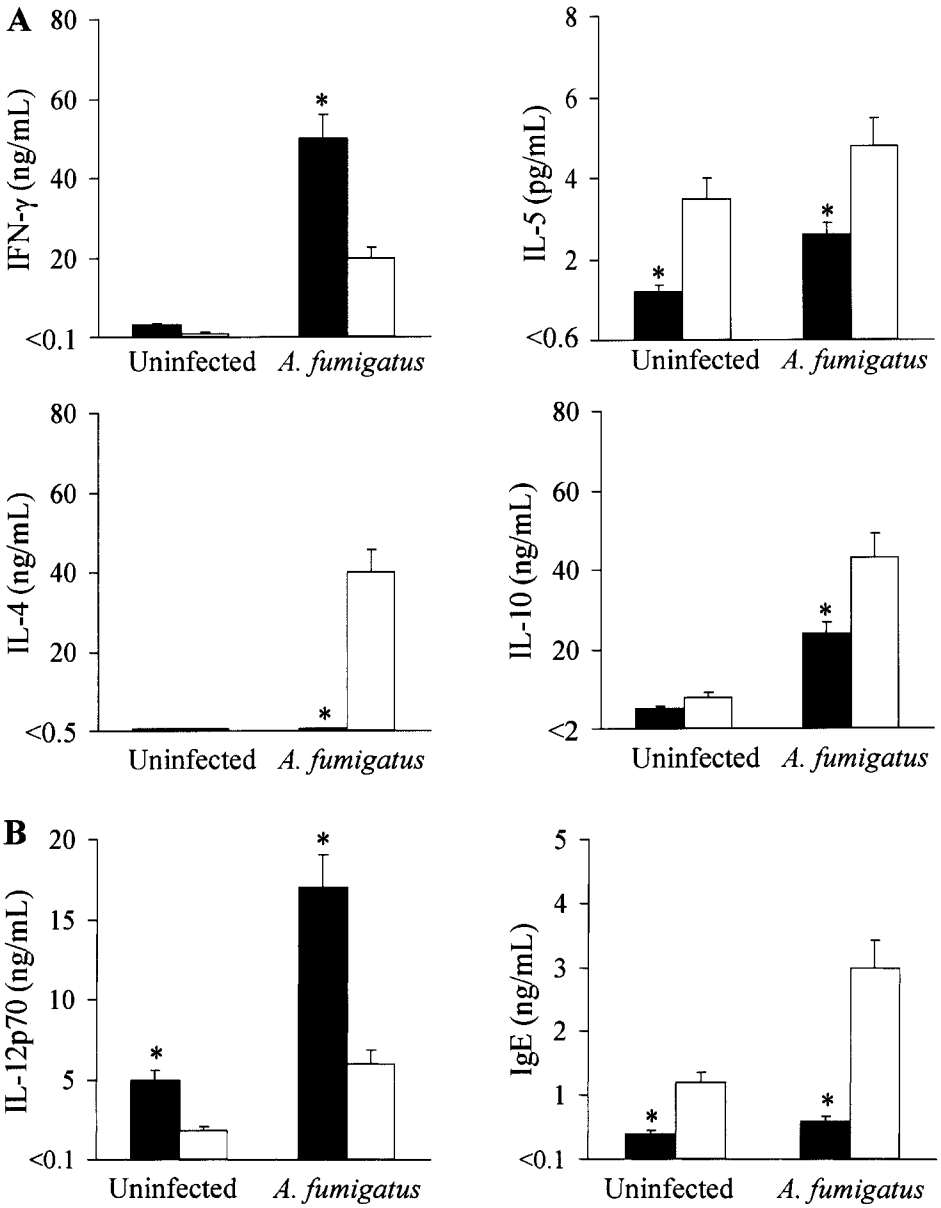

Figure 2. Cytokine production by lung lymphocytes *(A)* and interleukin (IL)–12p70 and IgE levels in bronchiolar lavage (BAL) fluid *(B)* from immunosuppressed IL-4–deficient (IL-$4^{-/-}$; solid bars) and –nondeficient (IL-$4^{+/+}$; open bars) mice with invasive pulmonary aspergillosis. Interstitial lung lymphocytes and BAL fluid were obtained from mice uninfected or 1 day after last intranasal inoculation of *Aspergillus fumigatus* conidia. Interstitial lymphocytes were stimulated with anti-CD3 for 48 h. Cytokine and antibody levels were determined by cytokine-specific ELISAs or by modified antibody-capture bioassay (IL-12p70). Data are mean ± SE of 3–5 separate experiments, each involving replicate measurements. <, below assay detection limit. *$P < .05$, IL-$4^{-/-}$ vs. IL-$4^{+/+}$ mice, Student's *t* test. IFN, interferon.

damage and susceptibility in wild-type mice, we evaluated the course and outcome of the infection in IL-$5^{-/-}$ mice, which lack eosinophils [28]. Of interest, IL-$5^{-/-}$ mice were as susceptible as wild-type mice to the infection (table 1). Histopathologic examination of lung sections from immunosuppressed IL-$5^{-/-}$ mice revealed patterns of lesions similar to those observed in IPA and non-IPA [4, 17]. The finding of a massive influx of neutrophils, but not eosinophils, in highly susceptible IL-$5^{-/-}$ mice (E.C., unpublished data) indicates that neutrophils, rather than eosinophils, contribute to lung pathology in IL-$4^{+/+}$ mice with IPA.

Th1 and Th2 cell activation in IL-$4^{-/-}$ and IL-$4^{+/+}$ mice with IPA. To correlate susceptibility and resistance of IL-$4^{+/+}$ and IL-$4^{-/-}$ mice to IPA with the activation of Th1 and Th2 cells in the lungs, we assessed Th cell polarization by measuring Th1 (IFN-γ) and Th2 (IL-4, -5, and -10) cytokine production in

culture supernatants of purified lung interstitial lymphocytes stimulated with anti-CD3. The levels of IL-12p70 and of total IgE were also measured in BAL fluid. Lung T cells from IL-4$^{-/-}$ mice produced (50%–60%) elevated levels of IFN-γ, whereas IL-5 and IL-10 levels were reduced by ~50% (figure 2*A*). Further measurement of IL-12p70 in the BAL fluid showed increased levels in IL-4$^{-/-}$ mice, which, as expected, had low levels of IgE (figure 2*B*). This indicates that IL-4 down-regulates IL-12 production, and consequently Th1 cell development, in lungs of susceptible BALB/c mice. Of interest, RT-PCR analysis of the IL-12R showed IL-12Rβ2 mRNA expression in interstitial lymphocytes of IL-4$^{-/-}$ mice but not in those of BALB/c wild-type mice, whereas IL-12Rβ1 mRNA was expressed at similar levels by both types of mice (figure 3). It has been suggested that expression of the IL-12Rβ2 chain may be down-regulated during Th2 cell development in BALB/c mice [36].

Next we assessed the role of Th1 responses during IPA and whether IL-4$^{-/-}$ mice develop resistance because of the augmented IL-12 levels and ensuing Th1 responses. To this end, *A. fumigatus* infection was evaluated in IL-12p40$^{-/-}$ and IFN-$\gamma^{-/-}$ mice and in BALB/c wild-type and IL-4$^{-/-}$ mice treated with IL-12 neutralizing antibody. Measurement of the chitin content in lungs, which correlates with fungus burden, showed that susceptible BALB/c mice became even more susceptible in the absence of IL-12 (by neutralization or by genetic disruption) and IFN-γ (figure 4*A*). Moreover, IL-4$^{-/-}$ mice treated with antibody to IL-12 developed susceptibility similar to that of IL-12$^{-/-}$ and IFN-$\gamma^{-/-}$ mice (figure 4*A*).

We next asked whether antifungal effector cells of IL-4$^{-/-}$ mice possess elevated activity. The results show that, soon after infection, the conidiocidal activity of macrophages does not differ between resistant IL-4$^{-/-}$ and susceptible wild-type BALB/c mice (figure 4*B*). In contrast, hyphal damage of neutrophils was increased by >80% in IL-4$^{-/-}$ compared with wild-type mice (figure 4*B*). Together, these results suggest that the resistance of IL-4$^{-/-}$ mice to IPA is mediated by elevated type 1 responses.

Costimulation dependency of Th cell activation in mice with IPA. To determine the contribution of the B7-1 and B7-2 costimulatory molecules in the development of antifungal Th responses, we evaluated the expression of these molecules in the lungs of mice upon infection and assessed the effects of their neutralization on Th cell polarization and the outcome of infection. Figure 5 shows that both molecules were expressed on B cells and phagocytic cells in IL-4$^{+/+}$ and, to a lesser extent, in IL-4$^{-/-}$ mice. Local treatment of BALB/c mice with anti–B7-1 MAb alone or with a combination of anti–B7-1 and anti–B7-2 MAbs greatly decreased fungus burden in the lung and concomitantly resulted in high-level production of IL-12 and IFN-γ, whereas IL-4 and IL-10 production was unaltered (table 2). Similar results were obtained by treatment with the CTLA-4 Ig molecule, which neutralizes B7-1 and B7-2 (E.C., unpublished data). In contrast, treatment of BALB/c mice with anti–B7-2 MAb alone resulted in impaired production of IL-4 and IL-10, while leaving IL-12 and IFN-γ levels unaltered (table 2). Of interest, fungus burden was slightly increased rather than decreased, despite reduced Th2 responses, a finding suggesting that the extent of fungal growth correlates with levels of Th1 rather than levels of Th2 immune reactivity. Similar results were obtained with antibody treatment of IL-4$^{-/-}$ mice (table 2). Anti–B7-1 MAb alone or a combination of anti–B7-1 and anti–B7-2 MAbs further increased type 1 cytokine (IL-12 and IFN-γ) production, which was already increased in these mice because of the absence of IL-4. As a result, fungus burden became even more reduced in resistant IL-4$^{-/-}$ mice. In contrast, anti–B7-2 treatment did not further decrease Th2 cytokine production (IL-10), which was already low due to the absence of IL-4. Together, these results strongly suggest that fungus burden correlates well with the intensity of type 1 but not type 2 responses and that IL-4$^{-/-}$ mice became resistant to IPA because of increased Th1 development rather than decreased Th2 development. Figure 6 shows a regression analysis of cytokine production (IL-12, IFN-γ, and IL-10) versus fungus burden (i.e., chitin content), which supports this conclusion from experimental findings.

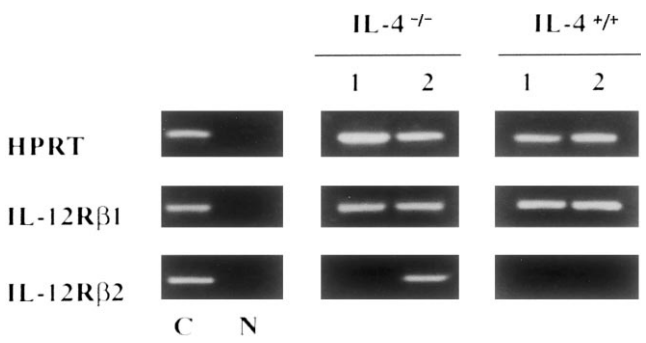

Figure 3. Expression of interleukin (IL)–12R genes in immunosuppressed IL-4–deficient (IL-4$^{-/-}$) and –nondeficient (IL-4$^{+/+}$) mice with invasive pulmonary aspergillosis. Total lung cells from mice uninfected (lane 1) or 1 day after last intranasal inoculation of *Aspergillus fumigatus* conidia (lane 2). Cytokine receptor gene expression was assessed by reverse-transcription polymerase chain reaction (PCR). C, hypoxanthine-guanine phosphoribosyl transferase (HPRT)– or cytokine receptor–specific controls; N, no DNA added to amplification mix during PCR.

Discussion

This study was undertaken to identify the precise mechanism(s) through which IL-4 exerts its pathogenetic role in mice with IPA. IL-4$^{-/-}$ mice were highly resistant to IPA compared with wild-type mice. Resistance was associated with decreased lung inflammatory pathology and fungal growth and local occurrence of IL-12–dependent, IFN-γ–producing Th1 cells. The results demonstrate that IL-4 is responsible for the suppression

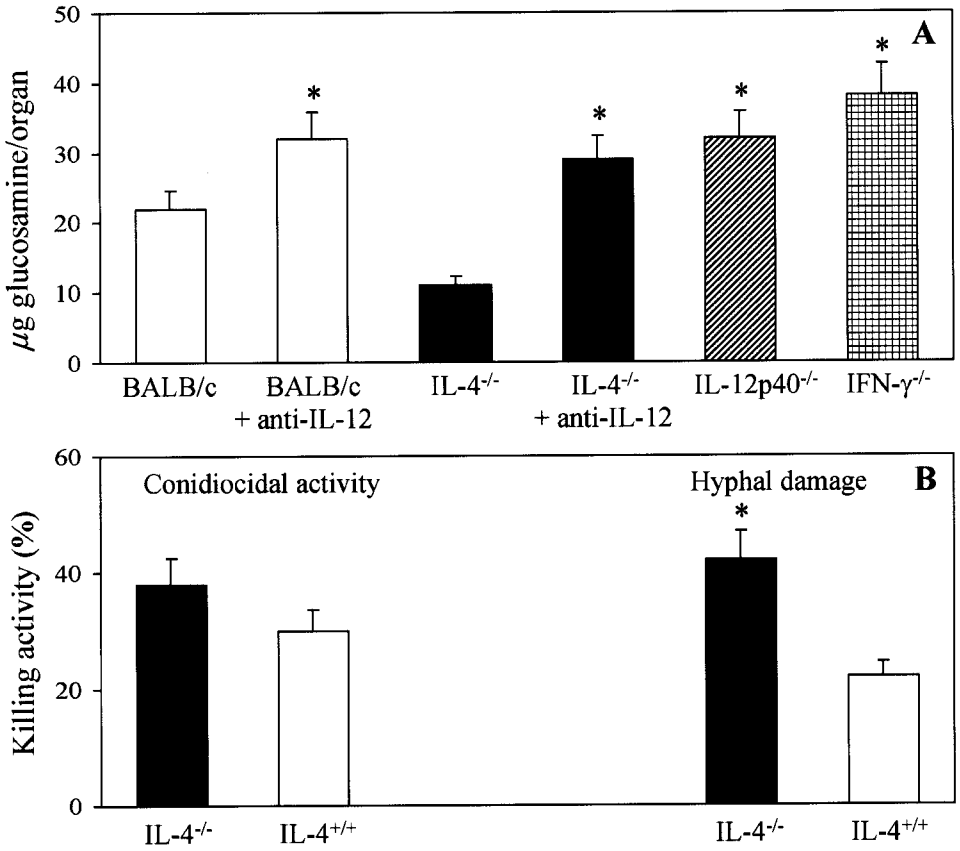

Figure 4. Invasive pulmonary aspergillosis (IPA) course in immunosuppressed BALB/c wild-type and interleukin-4–deficient (IL-$4^{-/-}$) mice upon IL-12 neutralization and in IL-$12p40^{-/-}$ and interferon-γ–deficient (IFN-$\gamma^{-/-}$) mice. *A,* for IL-12 neutralization, 500 μg of sheep anti–IL-12 antibody was given intraperitoneally 4 h before fungal inoculation. Fungal colonization in lung was determined 1 day after last intranasal (inl) inoculation of *Aspergillus fumigatus* conidia and is expressed as μg of glucosamine/organ (chitin content). *B,* conidiocidal activity and hyphal damage of purified macrophages and neutrophils, respectively, from IL-$4^{-/-}$ or IL-4–nondeficient (IL-$4^{+/+}$) mice with IPA. Conidiocidal activity and hyphal damage from uninfected IL-$4^{-/-}$ and IL-$4^{+/+}$ mice, expressed as %, were as follows: 22 ± 2 vs. 18 ± 3 and 25 ± 2 vs. 18 ± 4. Assays were done as described in Materials and Methods 1 day after last inl inoculation of *A. fumigatus* conidia. Values are mean ± SE of 3–5 experiments. *$P < .05$, treated vs. untreated mice and mutant vs. wild-type mice, Student's t test.

of protective Th1 cell responses, a finding in line with the curative effect observed on IL-4 inhibition in IL-$4^{+/+}$ mice [17, 30, 37].

The recruitment and activation of inflammatory cells in the setting of pathogen challenge is a complex and dynamic process that involves the coordinated expression of both pro- and anti-inflammatory mediators, such as chemokines and cytokines [38, 39]. Indeed, although an inflammatory response is essential to clear pathogens from the site of infection, a prolonged inflammatory response might worsen lung injury and interfere with pathogen elimination [40].

We found that, although the absolute numbers of cells recruited into the lungs were not different in IL-$4^{-/-}$ and IL-$4^{+/+}$ mice upon infection (E.C., unpublished data), the overall inflammatory response was mitigated in the absence of IL-4, as judged by the reduced number of inflammatory cells at the lesion sites. These results are not consistent with the anti-inflammatory activity of IL-4 in the lungs, which includes reduction of neutrophil recruitment and of synthesis of IL-8 [41]. However, in addition to its role in promoting IgE immediate-type hypersensitivity [19], IL-4 also has proinflammatory effects in fungal infections, such as enhancement of macrophage mannose receptor activity [42], stimulation of phagocytosis and killing of yeast cells [43, 44], and priming of neutrophils for IL-12 production [45]. Therefore, the effect IL-4 may have in the inflammatory lung response to fungi may be complex and may result from multiple activities. In fact, the decreased lung inflammatory response observed in IL-$4^{-/-}$ mice with IPA is at variance with that observed in IL-$4^{-/-}$ mice with ABPA [46–48]. In this model, no differences were observed in lung injury between IL-$4^{-/-}$ and IL-$4^{+/+}$ mice upon sensitization to *A. fumigatus* antigen, despite a characteristic Th1 response. Thus, IL-4 has a nonessential role in the development of lung injury during the allergic response to the fungus [46–48]. Moreover,

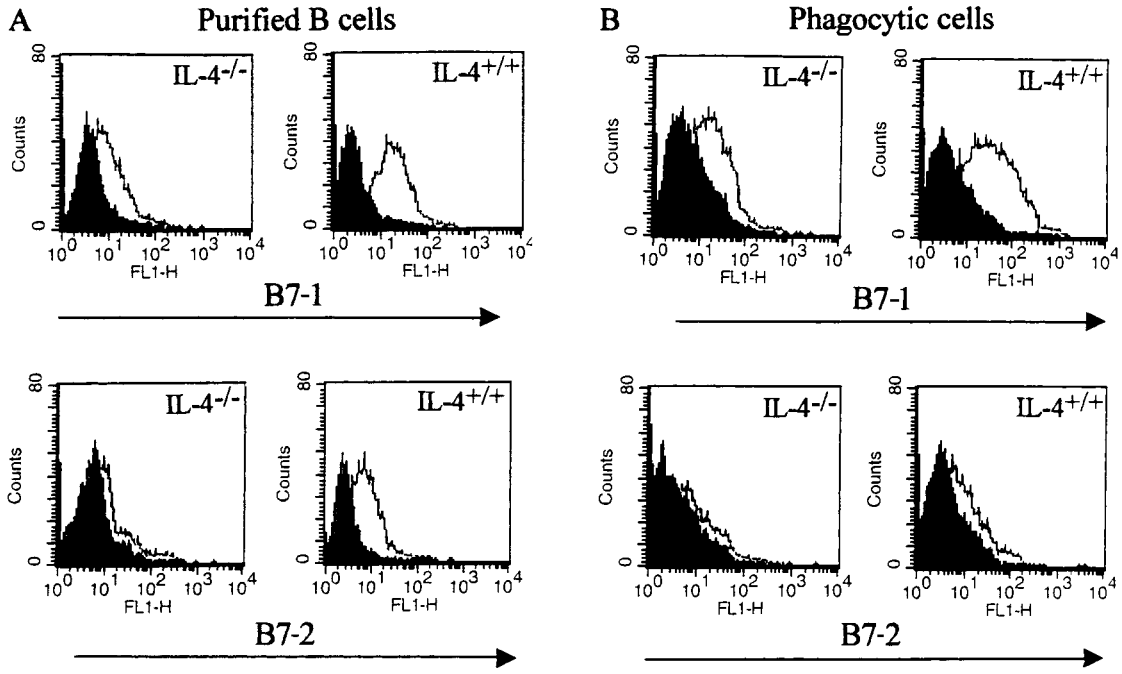

Figure 5. Expression of B7-1 and B7-2 costimulatory molecules in lungs of interleukin-4–deficient (IL-$4^{-/-}$) and –nondeficient (IL-$4^{+/+}$) mice with invasive pulmonary aspergillosis. Purified B *(A)* or total lung *(B)* cells recovered 1 day after last fungal challenge were stained with fluorescein isothiocyanate–conjugated anti–B7-1 or anti–B7-2 monoclonal antibodies (MAbs; open histogram) or irrelevant MAb (black histogram). Phagocytic cells were electronically gated by forward light and side scatter analysis.

although eosinophils were differentially recruited in the lungs of IL-$4^{-/-}$ and IL-$4^{+/+}$ mice upon infection, the finding that the inflammatory response and course of infection were not improved in IL-$5^{-/-}$ mice suggests a minimal role, if any, for eosinophils in the pathogenesis of IPA. These data indicate that cellular and molecular players of inflammation may have different roles in the pathogenesis of allergic and nonallergic lung diseases caused by *A. fumigatus.* In the case of invasive aspergillosis, neutrophils appear to be an important mediator of antifungal inflammatory and immune responses, a finding in line with that observed in candidiasis [49].

IL-4 has a pivotal role in CD4 Th2 differentiation in both allergic [19, 50] and nonallergic [17, 30, 37] diseases caused by *A. fumigatus.* IgE production was absent in IL-$4^{-/-}$ mice with ABPA [46], and IL-4 neutralization increased Th1-dependent protective immunity in mice with aspergillosis [17, 30, 37]. We found that susceptibility of IL-$4^{+/+}$ mice to IPA was associated with the activation of Th2 lymphocytes producing IL-4, IL-5, and IL-10 and with the production of IgE. In contrast, resistance of IL-$4^{-/-}$ mice to infection was associated with the production of IL-12 and the activation of interstitial T lymphocytes producing IFN-γ and responsive to IL-12. We recently reported that, in addition to IL-12 production, responsiveness to IL-12 is a prerequisite for Th1 cell development in mice with *Candida albicans* infections [51]. Similar conditions appear to be required for the generation of Th1 cell reactivity to *A. fumigatus,* as Th1-mediated resistance was impaired in IL-$4^{-/-}$ mice upon IL-12 neutralization and in IL-$12^{-/-}$ mice. Moreover, lung lymphocytes from IL-$4^{-/-}$, but not IL-$4^{+/+}$, mice expressed functional IL-12 receptor.

The finding that resistance to infection is impaired in IFN-$\gamma^{-/-}$ mice indicates the important role this cytokine may have in the control of *A. fumigatus* infection. In addition to its role in regulating the generation of antifungal Th1 cells [35], IFN-γ potentiates the oxidative responses and hyphal damage of human phagocytic cells [52], as observed in patients with chronic granulomatous disease treated with IFN-γ [53]. These observations are in line with our finding of the increased antifungal activity of neutrophils in infected IL-$4^{-/-}$ mice. Against *Aspergillus* species, Th1 (IFN-γ and TNF-α) and Th2 (IL-4 and IL-10) cytokines have opposite effects on phagocytic host defenses [5]. IL-10, more than IL-4, has a suppressive effect on the antifungal activity of mononuclear cells against the fungus [5]. It has been suggested that IL-10 may have a pathogenetic role in the chronic form of pulmonary aspergillosis that is independent of neutropenia and corticosteroid therapy [12, 14]. We previously showed that IL-10 inhibition early in infection ameliorates the course of IPA in highly susceptible mice [17]. In the present study, we found that IL-10 was produced in IL-$4^{+/+}$ mice upon infection and was elevated in conditions of increased susceptibility to the infection, such as those observed in IL-$12^{-/-}$ or IFN-$\gamma^{-/-}$ mice or in IL-$4^{-/-}$ mice upon IL-12

Table 2. Effect of B7-1 and B7-2 neutralization on fungal growth and cytokine production in interleukin (IL)–$4^{+/+}$ and IL-$4^{-/-}$ mice with invasive pulmonary aspergillosis.

Mice and treatment[a]	Chitin content[b]	Cytokine[c]			
		IL-12p70	IFN-γ	IL-4	IL-10
IL-$4^{+/+}$					
Neither	29 ± 2.7	5 ± 2	14 ± 3	44 ± 3	61 ± 4
Anti–B7-1	14 ± 3.0[d]	24 ± 3[d]	84 ± 4[d]	55 ± 6	50 ± 6
Anti–B7-2	39 ± 4.1[d]	7 ± 1	17 ± 2	18 ± 2[d]	26 ± 5[d]
Both	18 ± 2.1[d]	21 ± 2[d]	71 ± 3[d]	37 ± 5	47 ± 3
IL-$4^{-/-}$					
Neither	13 ± 3.5	18 ± 2	68 ± 5	<0.5	18 ± 4
Anti–B7-1	7 ± 0.5[d]	28 ± 4[d]	110 ± 6[d]	<0.5	21 ± 5
Anti–B7-2	20 ± 3.3	22 ± 4	58 ± 4	<0.5	14 ± 3
Both	9 ± 1.7[d]	32 ± 3[d]	98 ± 3[d]	<0.5	16 ± 2

NOTE. IL-$4^{+/+}$, IL-4 nondeficient; IL-$4^{-/-}$, IL-4 deficient; IFN, interferon.

[a] Mice were treated with anti–B7-1 and/or anti–B7-2 monoclonal antibody (given intranasally [inl] at 100 μg/50 μL) 4 h before *Aspergillus fumigatus* injection.

[b] Chitin content in lungs, expressed as μg of glucosamine/organ, was determined 3 days after last fungal inl inoculation. Values are mean ± SE of 3–5 separate experiments.

[c] Cytokine levels (ng/mL) in bronchoalveolar lavage fluid (IL-12p70) or in culture supernatants of anti-CD3–stimulated lung T cells were determined 3 days after last fungal inoculation. Values are mean ± SE of 3–5 separate experiments, each involving replicate measurements. <0.5, below assay detection limit.

[d] $P < .05$ (treated vs. untreated mice, Student's t test).

neutralization (E.C., unpublished data). However, these results, although suggestive of a detrimental effect of IL-10 in mice with IPA, do not allow us to predict its precise role in infection, given the potentially complex role of IL-10 in the pathogenesis of lung pathology and infections [26, 38, 54–56].

The mechanisms underlying the activation of Th cells in the lungs of mice with IPA are not yet defined. The type of Th cell responses to antigens encountered on the mucosal surfaces of the respiratory tract may be influenced by regional environmental factors, such as the nature of regional innate immunity [57] and the expression of costimulatory molecules [58–60]. On looking at the expression of B7-1 and B7-2 molecules in the lungs of IL-$4^{+/+}$ and IL-$4^{-/-}$ mice upon infection, we found an increased expression of both costimulatory molecules in the lungs of IL-$4^{+/+}$ mice. In contrast, the expression of B7-1 and B7-2, in particular, was low in IL-$4^{-/-}$ mice, consistent with the finding that IL-4 can induce expression of both costimulatory molecules on B cells [61]. More importantly, blockade of either of the costimulatory molecules had a different effect on fungus burden and pattern of Th cytokine production in either type of mice. B7-1 neutralization was followed by decreased fungal growth and elevated production of both IL-12 and IFN-γ, whereas levels of IL-4 and IL-10 were not substantially modified. In contrast, B7-2 neutralization appeared to reduce Th2 cytokine production without affecting that of IL-12 and IFN-γ. This pattern of cytokine production is consistent with the results obtained on fungal growth. Indeed, cytokines with activating signals for fungicidal effector cells were increased upon B7-1 neutralization. In contrast, cytokines with deactivating signals were down-regulated upon B7-2 neutralization, but, as a result of B7-1 engagement, cytokines with activating signals were not increased. This finding may explain why the fungal growth was not decreased in these mice. In fact, the fungal growth was somewhat increased upon B7-2 neutralization, particularly in IL-$4^{+/+}$ mice.

Whether B7-2 has an effect on fungal growth beyond its costimulatory activity remains to be determined. The finding that simultaneous blockade of both molecules led to effects similar to those obtained upon B7-1 blockade indicates a dominant role of B7-1 over B7-2 in the development of antifungal immunity in mice with IPA. In particular, as no evidence of immune deviation was observed during each treatment, it appears that each costimulatory molecule may have a distinct, nonoverlapping role in the antifungal immune response in vivo. Because IL-12 and IFN-γ production were increased upon B7-1 neutralization, the engagement of the B7-1 molecule appears

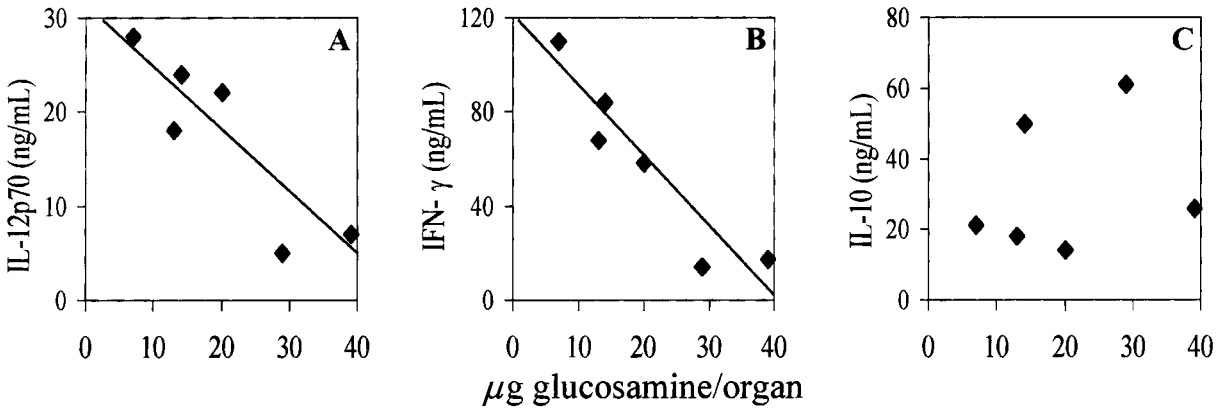

Figure 6. Correlation of fungal load with T helper (Th) 1 (interleukin [IL]–12p70 and interferon [IFN]–γ) but not Th2 (IL-10) cytokine production in mice with invasive pulmonary aspergillosis. Regression analysis was performed by plotting values of chitin content (μg of glucosamine/organ) as function of IL-12p70 *(A)*, IFN-γ *(B)*, or IL-10 *(C)* production. Data are from table 2. Linear regression analysis showed correlation coefficients (r^2) of .78, .88, and .62 (*A–C*, respectively).

to result in an inhibitory signal for IL-12 and IFN-γ production. That B7-1 may provide both a costimulatory and a down-regulatory signal during the course of an immune response has been reported [62, 63]. In our study, the blockade of the CTLA-4 inhibitory coreceptor also resulted in the activation of the IL-12– and IFN-γ–dependent pathway (data not shown), thus strongly indicating that the down-regulatory signal delivered by B7-1 may occur through its engagement with the CTLA-4 coreceptor, as reported [64]. Although studies in CTLA-4– or CD28-deficient mice will definitively clarify this issue, it appears that one important role of B7-1 in the activation of B7-2–dependent Th2 antifungal responses is the concomitant inhibition of IL-12–dependent Th1 reactivity. This would corroborate the suggestion that the amount of T cell costimulatory signals in the form of B7-2 in nonlymphoid tissues may be limiting, leading to the need for further signaling provided by B7-1 [65].

Our findings would help to explain some still controversial results on the role of costimulatory molecules in the development of lung mucosal immunity. It is well documented that the induction of CD4 Th2 reactivity to aeroallergens is strictly dependent on B7-2, but not B7-1, costimulatory molecules [58, 59]. However, in infection with *Bordetella pertussis,* hyporesponsiveness of lung T cells is associated with a biased expression of B7-1 in relation to B7-2 [60]. These results parallel those obtained in infection by the nematode parasite *Heligmosomoides polygyrus,* in which blockade of both costimulatory molecules was required to inhibit the parasite-specific Th2 immune response [66, 67]. Thus, it is clear that the requirement for costimulatory molecules during immune responses is quite complex and likely depends on a number of factors unique to each specific type of immune response. In particular, the role of costimulation may vary in the different lymphoid microenvironments, paradoxes being observed in antigen-specific mucosal and systemic immune responses in CTLA4-Hg1 transgenic mice [65].

All together, the results of the present study suggest a possible scenario of IL-4 regulation of costimulation-dependent Th immunity in mice with IPA. In addition to its growth-promoting activity on Th2 cells, IL-4 appears to be required, although not essential, for local expression of both costimulatory molecules, whose engagement results in up-regulation of IL-4 and IL-10 and concomitant down-regulation of IFN-γ and IL-12. This would predict that, in conditions of functional IL-4 deficiency, the defective expression of both molecules will further contribute to the decreased production of Th2 cytokines, such as IL-10, while increasing that of IFN-γ and IL-12. It appears that the balance between Th1 and Th2 cells in mice with IPA is determined by many induction pathways and that costimulation and promotion of Th2 cell growth are successfully controlled by IL-4. Experiments in which exogenous IL-4 is given to IL-$4^{-/-}$ mice will clarify this issue.

In conclusion, our results demonstrate that IL-$4^{-/-}$ mice are resistant to IPA and its pathology through the induction of protective Th1 responses and that the induction of lung effector Th2 responses in allergy and infection may occur through distinct costimulatory pathways. IL-4 appears to be responsible for the inhibition of Th1 cell development and for the generation of Th2 cell responses. However, Th2 effector mechanisms (including eosinophils) per se do not critically increase susceptibility to the infection, a finding in line with the observation that atopy is a predisposing factor for developing ABPA [50] but not invasive aspergillosis [4] in humans. Because mortality with invasive aspergillosis is very high, the present study is an effort to better understand host defenses against *A. fumigatus,* with the ultimate goal of improving management of an infection with such a dismal outcome.

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References

1. Rhodes JC. Aspergillosis. In: Murphy JW, Friedman H, Bendinelli M, eds. Fungal infections and immune responses. New York: Plenum Press, **1993**: 359–77.
2. Saral R. *Candida* and *Aspergillus* infections in immunocompromised patients: an overview. Rev Infect Dis **1991**;13:487–92.
3. Binder RE, Faling LJ, Pugatch RD, Mahasen C, Snider GL. Chronic necrotizing pulmonary aspergillosis: a discrete clinical entity. Medicine **1982**;61:109–24.
4. Denning DW. Invasive aspergillosis. Clin Infect Dis **1998**;26:781–805.
5. Roilides E, Katsifa H, Walsh TJ. Pulmonary host defences against *Aspergillus fumigatus.* Res Immunol **1998**;149:454–65.
6. Schaffner A, Douglas H, Braude A. Selective protection against conidia by mononuclear and against mycelia by polymorphonuclear phagocytes in resistance to *Aspergillus:* observations on these two lines of defense in vivo and in vitro with human and mouse phagocytes. J Clin Invest **1982**;69: 617–31.
7. Cohen MS, Isturiz RE, Malech HL, et al. Fungal infection in chronic granulomatous disease: the importance of the phagocytes in defense against fungi. Am J Med **1981**;71:59–66.
8. Morgenstern DE, Gifford MAC, Li LL, Doerschuk CM, Dinauer MC. Absence of respiratory burst in X-linked chronic granulomatous disease mice leads to abnormalities in both host defense and inflammatory response to *Aspergillus fumigatus.* J Exp Med **1997**;185:207–18.
9. Schnaffer A. Therapeutic concentrations of glucocorticoids suppress the antimicrobial activity of human macrophages without impairing their responsiveness to gamma interferon. J Clin Invest **1985**;76:1755–64.
10. Palmer LB, Greenberg HE, Schiff M. Corticosteroid treatment as a risk factor for invasive aspergillosis in patients with lung disease. Thorax **1991**;46: 15–20.
11. Gerson SL, Talbot GH, Hurwitz S, Strom BL, Lusk EJ, Cassileth PA. Prolonged granulocytopenia: the major risk factor for invasive pulmonary aspergillosis in patients with acute leukemia. Ann Intern Med **1984**;100: 345–51.
12. Denning DW, Follansbee SE, Scolaro M, Norris S, Edelstein H, Stevens DA. Pulmonary aspergillosis in the acquired immunodeficiency syndrome. N Engl J Med **1991**;324:654–62.
13. Roilides E, Holmes A, Blake C, Pizzo PA, Walsh TJ. Impairment of neutrophil antifungal activity against hyphae of *Aspergillus fumigatus* in chil-

dren infected with human immunodeficiency virus. J Infect Dis **1993**; 167: 905–11.

14. Roilides E, Dimitriadou A, Kadiltsoglou I, et al. IL-10 exerts suppressive and enhancing effects on antifungal activity of mononuclear phagocytes against *Aspergillus fumigatus.* J Immunol **1997**; 158:322–9.
15. Grazziutti ML, Rex JH, Cowart RE, Anaissie EJ, Ford A, Savary CA. *Aspergillus fumigatus* conidia induce a Th1-type cytokine response. J Infect Dis **1997**; 176:1579–83.
16. Wolach B, Alon E, Gottesman G, Yellin A. Pulmonary aspergillosis in a child with hyperimmunoglobulin E syndrome. Clin Infect Dis **1998**; 26:204–5.
17. Cenci E, Mencacci A, Fè d'Ostiani C, et al. Cytokine- and T helper–dependent lung mucosal immunity in mice with invasive pulmonary aspergillosis. J Infect Dis **1998**; 178:1750–60.
18. Romani L. The T cell response against fungal infections. Curr Opin Immunol **1997**; 9:484–90.
19. Ryan JJ. Interleukin-4 and its receptor: essential mediators of the allergic response. J Allergy Clin Immunol **1997**; 99:1–5.
20. Foster PS, Hogan SP, Ramsay AJ, Matthaei KI, Young IG. Interleukin 5 deficiency abolishes eosinophilia, airway hyperreactivity, and lung damage in a mouse asthma model. J Exp Med **1996**; 183:195–201.
21. Corry DB, Folkesson HG, Warnock ML, et al. Interleukin 4, but not interleukin 5 or eosinophils, is required in a murine model of acute airway hyperreactivity. J Exp Med **1996**; 183:109–17.
22. Horgan SP, Mould A, Kikutani H, Ramsay AJ, Foster PS. Aeroallergen-induced eosinophilic inflammation, lung damage, and airways hyperreactivity in mice can occur independently of IL-4 and allergen-specific immunoglobulins. J Clin Invest **1997**; 99:1329–39.
23. Kurup VP, Mauze S, Choi S, Seymour BW, Coffman RB. A murine model of allergic bronchopulmonary aspergillosis with elevated eosinophils and IgE. J Immunol **1992**; 148:3783–8.
24. Knutsen AP, Mueller KR, Levine AD, Choulan B, Hutcheson PS, Slavin RG. *Asp*fI $CD4^+$ Th2-like T-cell lines in allergic bronchopulmonary aspergillosis. J Allergy Clin Immunol **1994**; 94:215–21.
25. Crameri R, Faith A, Hemmann S, et al. Humoral and cell-mediated autoimmunity in allergy to *Aspergillus fumigatus.* J Exp Med **1996**; 184:265–70.
26. Grünig G, Corry DB, Leach MV, Seymour BWP, Kurup VP, Rennick BM. Interleukin-10 is a natural suppressor of cytokine production and inflammation in a murine model of allergic bronchopulmonary aspergillosis. J Exp Med **1997**; 185:1089–99.
27. Kopf M, Le Gros G, Bachmann M, Lamers MC, Bluethmann H, Kohler G. Disruption of the murine IL-4 gene blocks Th2 cytokine responses. Nature **1993**; 362:245–8.
28. Kopf M, Brombacher F, Hodgkin PD, et al. IL-5 deficient mice have a developmental defect in $CD5^+$ B-1 cells and lack eosinophilia but have normal antibody and cytotoxic T cell responses. Immunity **1996**; 4:15-24.
29. Dalton DK, Pitts MS, Keshaw S, Figari IS, Bradley A, Stewart TA. Multiple defects of immune cell function in mice with disrupted interferon-gamma genes. Science **1993**; 259:1739–42.
30. Cenci E, Perito S, Enssle KH, et al. Th1 and Th2 cytokines in mice with invasive aspergillosis. Infect Immun **1997**; 65:564–70.
31. Razi-Wolf Z, Galvin F, Gary G, Reiser H. Evidence for an additional ligand, distinct from B7, for the CTLA-4 receptor. Proc Natl Acad Sci USA **1993**; 90:11182–6.
32. Hathcock KS, Laszlo G, Dickler HB, Bradshaw J, Linsley P, Hodes RJ. Identification of an alternative CTLA-4 ligand costimulatory for T cell activation. Science **1993**; 262:905–7.
33. Romani L, Mencacci A, Cenci E, Del Sero G, Bistoni F, Puccetti P. An immunoregulatory role for neutrophils in $CD4^+$ T helper subset selection in mice with candidiasis. J Immunol **1997**; 158:2356–62.
34. Mencacci A, Cenci E, Del Sero G, et al. Defective costimulation and impaired Th1 development in TNF/LT-α-double deficient mice infected with *Candida albicans.* Int Immunol **1998**; 10:37–48.
35. Cenci E, Mencacci A, Del Sero G, et al. IFN-γ is required for IL-12 responsiveness in mice with *Candida albicans.* J Immunol **1998**; 161:3543–50.
36. Szabo SJ, Dighe AS, Gubler U, Murphy KM. Regulation of the interleukin (IL)-12Rβ2 subunit expression in developing T helper 1 (Th1) and Th2 cells. J Exp Med **1997**; 185:817–24.
37. Cenci E, Mencacci A, Fè d'Ostiani C, et al. Cytokine- and T-helper–dependent immunity in murine aspergillosis. Res Immunol **1998**; 149: 445–54.
38. Huffnagle GB, Lipscomb MF. Cells and cytokines in pulmonary cryptococcosis. Res Immunol **1998**; 149:387–96.
39. Kolls JK, Nelson S, Summer WR. Recombinant cytokines and pulmonary host defense. Am J Med Sci **1993**; 306:330–5.
40. Smith JA. Neutrophils, host defense, and inflammation: a double-edged sword. J Leukoc Biol **1994**; 56:672–86.
41. Wertheim WA, Kunkel SL, Standiford TJ, et al. Regulation of neutrophil-derived IL-8: the role of prostaglandin E2, dexamethasone, and IL-4. J Immunol **1993**; 151:2166–75.
42. Stein M, Keshav S, Harris N, Gordon S. Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. J Exp Med **1992**; 176:287–92.
43. Redmond H, Schuchter L, Shou R, et al. Interleukin 4 (IL-4) enhances macrophage microbicidal function. J Leukoc Biol **1990**; 48(suppl):24.
44. Bobert LA, Waters TA, Pugliese-Sivo CC, Sullivan LM, Narula SK, Grace MJ. IL-4 induces neutrophilic maturation of HL-60 cells and activation of human peripheral blood neutrophils. Clin Exp Immunol **1995**; 99: 129–36.
45. Mencacci A, Del Sero G, Cenci E, et al. IL-4 is required for development of protective $CD4^+$ T helper type 1 cell responses to *Candida albicans.* J Exp Med **1998**; 187:307–17.
46. Kurup VP, Guo J, Murali PS, Choi H, Fink JN. Immunopathologic responses to *Aspergillus* antigen in IL-4 knockout mice. J Lab Clin Med **1997**; 130: 567–75.
47. Kurup VP, Choi HY, Murali PS, Xia JQ, Coffman RL, Fink JN. Immune responses to *Aspergillus* antigen in IL-$4^{-/-}$ mice and the effect of eosinophil ablation. Allergy **1999**; 54:420–7.
48. Kurup VP, Xia JQ, Rickaby BA, Dawson CA, Choi H, Fink JN. *Aspergillus fumigatus* antigen exposure results in pulmonary lung airway resistance in wild-type but not in IL-4 knockout mice. Clin Immunol **1999**; 90: 404–10.
49. Romani L, Bistoni F, Puccetti P. Initiation of T-helper cell immunity to *Candida albicans:* the role of neutrophils. Chem Immunol **1997**; 68:110–35.
50. Kurup VP, Grunig G, Knutsen AP, Murali PS. Cytokines in allergic bronchopulmonary aspergillosis. Res Immunol **1998**; 149:466–77.
51. Mencacci A, Cenci E, Bistoni F, et al. Specific and nonspecific immunity to *Candida albicans:* a lesson from genetically modified animals. Res Immunol **1998**; 149:352–61.
52. Roilides E, Uhlig K, Venzon D, Pizzo PA, Walsh TJ. Enhancement of oxidative response and damage caused by human neutrophils to *Aspergillus fumigatus* hyphae by granulocyte colony-stimulating factor and gamma interferon. Infect Immun **1993**; 61:1185–93.
53. Rex JH, Bennet JE, Gallin JI, Malech HL, DeCarlo ES, Melnick DA. In vivo interferon-γ therapy augments the in vitro ability of chronic granulomatous disease neutrophils to damage *Aspergillus* hyphae. J Infect Dis **1991**; 163:849–52.
54. Greenberger MJ, Strieter RM, Kunkel SL, Danforth JM, Goodman RE, Standiford TJ. Neutralization of IL-10 increases survival in a murine model of *Klebsiella* pneumonia. J Immunol **1995**; 155:722–9.
55. Sawa T, Corry DB, Gropper MA, Ohara M, Kurahashi K, Wiener-Kronish JP. IL-10 improves lung injury and survival in *Pseudomonas aeruginosa* pneumonia. J Immunol **1997**; 159:2858–66.
56. van der Poll T, Marchant A, Keogh CV, Goldman M, Lowry SF. IL-10 impairs host defense in murine pneumococcal pneumonia. J Infect Dis **1996**; 174:994–1000.
57. Lin JY, Wang Li F, Lin RH. The association between lung innate immunity and differential airway antigen-specific immune responses. Int Immunol **1996**; 8(4):499–507.

58. Tsuyuki S, Tsuyuki J, Einsle K, Kopf M, Coyle AJ. Costimulation through B7-2 (CD86) is required for the induction of a lung mucosal T helper cell 2 (Th2) immune response and altered airway responsiveness. J Exp Med **1997**; 185:1671–9.

59. Keane-Myers A, Gause WC, Linsley PS, Chen SJ, Wills-Karp M. B7-CD28/CTLA-4 costimulatory pathways are required for the development of T helper cell 2-mediated allergic airway responses to inhaled antigens. J Immunol **1997**; 158:2042–9.

60. McGuirk P, Mahon BP, Griffin F, Mills KHG. Compartmentalization of T cell responses following respiratory infection with *Bordetella pertussis:* hyporesponsiveness of lung T cells is associated with modulated expression of the co-stimulatory molecule CD28. Eur J Immunol **1998**; 28:153–63.

61. Stack RM, Lenschow DJ, Gray GS, Bluestone JA, Fitch FW. IL-4 treatment of small splenic B cells induces costimulatory molecules B7-1 and B7-2. J Immunol **1994**; 152:5723–33.

62. Sethna MP, Parijs LW, Sharpe AH, Abbas AK, Freeman GJ. A negative regulatory function of B7 revealed in B7-1 transgenic mice. Immunity **1994**; 1:415–21.

63. Gause CW, Halvorson MJ, Lu P, et al. The function of costimulatory molecules and the development of IL-4–producing T cells. Immunol Today **1997**; 18:115–20.

64. Fallarino F, Fields PE, Gajewski TF. B7-1 engagement of cytotoxic T lymphocyte antigen 4 inhibits T cell activation in the absence of CD28. J Exp Med **1998**; 188:205–10.

65. Gärdby E, Lane P, Lycke NY. Requirements for B7-CD28 costimulation in mucosal IgA responses: paradoxes observed in CTLA4-Hγ1 transgenic mice. J Immunol **1998**; 161:49–59.

66. Greenwald RJ, Lu P, Halvorson MJ, et al. Effects of blocking B7-1 and B7-2 interactions during a type 2 in vivo immune response. J Immunol **1997**; 158:4088–96.

67. Subramanian G, Katzura JW, Pearlman E, Jia X, Malhotra I, King CL. B7-2 requirement for helminth-induced granuloma formation and CD4 type 2 helper cell cytokine expression. J Immunol **1997**; 158:5914–20.